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BIOSYNTHESIS AND PURIFICATION OF V AND W ANTIGEN IN *PASTEURELLA PESTIS*[1]

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Burrows and Bacon demonstrated that virulent strains of *Pasteurella pestis* (1, 2) and *Pasteurella pseudotuberculosis* (3) produce two antigens, designated V and W, that are not produced by most avirulent strains. V^+W^+ strains were resistant to phagocytosis (4) and vaccination of mice with a V^+W^+ strain gave better protection against plague than vaccination with a V^-W^- strain (5). Direct evidence on the activity of each antigen depends on their separation and purification. This report summarizes our investigation into the biosynthesis, separation, purification and protective value of V and W antigens.

MATERIALS AND METHODS

Strains. P. pestis strain M23 (6) was chosen because it appeared to produce as much V and W antigens as any other virulent strain, but did not produce detectable amounts of Fraction I, a protein-carbohydrate complex usually present in large quantities in the supernatant fluid of typical virulent strains of *P. pestis*. *P. pestis* strain Alexander was used to challenge animals in passive protection experiments.

Quantitation of antigens. V and W antigen were assayed by diffusing various dilutions of a sample against a standard V or W antiserum in a gel plate prepared as follows. Twenty-five milliliters of 1% Ionagar no. 2 (Oxoid, Consolidated Laboratories, Chicago Heights, Illinois) in 0.9% NaCl, to which 10^{-4} Merthiolate had been added, was poured into a Petri plate. A plastic template, 10 mm thick, in which an exact pattern of holes had been drilled, was used to guide a steel punch in order to obtain equidistant holes in the agar. The holes were 5 mm in diameter and 2 mm from the edge of one hole to the edge of the adjacent hole. One-tenth milliliter of antigen or antiserum was placed in each hole and the results were recorded after incubation for 24 hr at 37°C. A pool of specific rabbit anti-V serum and a pool of specific rabbit anti-W serum (both obtained by absorption of all other antibodies) were frozen in small quantities, and a sample was thawed periodically to be used as a "standard" antiserum. The standard antiserum was placed in the center hole and 2-fold dilutions of antigen were placed in the six surrounding holes. This method of quantitating antigen was similar to the methods of Feinberg (7) and Thorne and Belton (8). A unit of antigen was arbitrarily considered to be the smallest amount of antigen that showed a visible band of precipitate between the antigen and antibody holes. Units were expressed as the reciprocal of the titer of the antigen. For example, an antigen preparation that showed a band of precipitate at a 1:8 dilution but was too weak to show a band at 1:16 contained 8 units of antigen/ml.

Chemical and physical assays. Protein was measured either by the spectrophotometric method, which relates protein concentration to the absorption of light at 215 mμ (9), or the chemical method of Lowry *et al.* (10). Carbohydrate was measured by the diphenylamine method (11). Lipid was estimated by use of the method of Snyder and Stephens (12). Nucleic acid was estimated from the extinction coefficient at 260 and 280 mμ (13). Molecular weights were calculated by the gel diffusion method of Polson (14).

RESULTS

V and W antigens are produced at 37°C but not at 26°C (?). However, at 37°C, avirulent V^-W^- mutants are favored in many media (15), resulting in little or no V and W antigen production. To avoid this, we grew strain M23 at 26°C in Difco heart infusion broth for 24 hr,

[1] In conducting the research reported herein, the investigators adhered to *Principles of Laboratory Animal Care* as established by the National Society for Medical Research.

sedimented the cells, and resuspended them at 36°C for 16 hr under various conditions. This study resulted in the following conclusions:

1. Bacto Casitone (at least 3%) was the best source of amino acids for the synthesis of V and W antigen.

2. Under these conditions, at least 0.01 M gluconate was essential. Ammonium or sodium gluconate were equally effective, and magnesium gluconate doubled the production of V and W antigen. It has been demonstrated by Brubaker and Surgalla (16) that, under different cultural conditions, energy sources other than gluconate can support the production of V and W antigen.

3. Calcium inhibited the synthesis of V and W antigen. This observation has been confirmed and extended by Brubaker and Surgalla (16).

4. No synthesis of V or W antigen occurred in an atmosphere of 100% CO_2 or 95% N_2-5% CO_2, or *in vacuo*.

Production of antigens for purification. On the basis of these findings, strain M23 was grown in Difco heart infusion broth at 26°C; the cells were sedimented, resuspended in a medium (300 ml/2-L flask) of 5% Bacto Casitone, 0.04 M sodium gluconate, and 0.04 M magnesium sulfate, and shaken at 36°C for 16 hr. During the incubation period, the viable number of microorganisms remained at 1 to 2 × 10^9/ml, and the pH remained near 7.0. The contents of the flasks were pooled and the cells permitted to settle overnight at 5°C. The clear supernatant fluid was siphoned off, filtered through sintered glass, and brought to 3.0 M with solid ammonium sulfate. The precipitated proteins were collected after standing approximately ½ hr, pH 6, 5°C, resuspended in a minimum amount of distilled water, and dialyzed overnight against cold distilled water. This procedure resulted in a 10-fold concentration and a 4- to 5-fold purification of both V and W antigen.

Purification, ammonium sulfate fractionation. A sample of sterile supernatant fluid that had been concentrated by ammonium sulfate precipitation and dialyzed, was used to determine the effectiveness of fractional increases of ammonium sulfate on the purification of V and W antigen. A 10-ml sample (1.1 units V and W antigen/mg of protein) was brought to 1.2 M by the addition of 3.5 M ammonium sulfate, pH 7.0, held 10 min, and centrifuged at 27,000 × G for 5 min. The precipitate was resuspended in distilled H_2O and tested for V, W and total protein. The supernatant fluid was brought to 1.4 M and the procedure repeated until 2.2 M ammonium sulfate was reached. The results (Table I) indicated that both V and W antigen were precipitated between 1.2 and 2.2 M, with the maximum purification occurring at 1.8 M (6-fold purification).

A repetition of this experiment, with the exception that each additional increment of ammonium sulfate was allowed to equilibrate with the sample overnight at 5°C, gave the same results.

The remainder of the batch was brought to 1.4 M ammonium sulfate, the precipitate removed, and the supernatant fluid brought to 2.2 M ammonium sulfate. Sixty-three per cent of both V and W antigen were recovered in the 2.2 M precipitate with a 4.5-fold purification. Since considerable quantities of V and W were precipitated at 1.4 M, subsequent batches were precipitated between 1.3 and 2.2 M ammonium sulfate.

Cellulose chromatography. The use of diethylaminoethyl (DEAE)-cellulose provided a

TABLE I

Fractionation of V and W antigen with ammonium sulfate (10-min equilibration)

Molarity[a] of Ammonium Sulfate	V		W		Total Protein
	Total Units	Units/Mg of Protein	Total Units	Units/Mg of Protein	
					mg
1.2	20	0.3	10	0.1	67.0
1.4	20	0.8	40	1.5	26.0
1.6	40	2.6	80	5.2	15.2
1.8	80	6.7	80	6.7	12.0
2.0	80	3.6	80	3.6	22.5
2.2	20	2.5	20	2.5	8.0
(2.2 Supernatant fluid)	0	0	0	0	72.2
Totals	260		310		223
Original material	320	1.1	320	1.1	296

[a] Original material was brought to the molarity indicated by the addition of 3.5 M ammonium sulfate, pH 7.0.

method of separating V from W antigen, and demonstrated a slight purification of V and a high purification of W antigen (Table II). The greatest adsorption of V antigen occurred when the sample was dialyzed against distilled water and then placed on DEAE-cellulose that had been washed with 1 N NaOH, neutralized with 0.018 M potassium phosphate buffer, pH 6.8, and thoroughly rinsed with distilled water. The difference in the percentages of V antigen recovered may be attributed to oversaturating the column in some cases. The column was washed stepwise with distilled water, 0.1 M, 0.3 M, and 0.5 M NaCl. The eluate flowed through a silica cell, and an estimation of the protein content was made with an improvised recording spectrophotometer consisting of a Bausch and Lomb monochromatic light source (set at 280 mμ) and a photosensitive tube connected to a Brown recorder. Each fraction was eluted until the recorder indicated that the eluate contained no protein. Depending on the ratio of protein to cellulose, a portion of the V antigen, but never W antigen, could be found in the distilled water wash. All of the V antigen that adsorbed to the column, but none of the W antigen, was eluted with 0.1 M NaCl. The 0.3 M NaCl eluate contained a large amount of protein, but no V antigen, and usually little or no W antigen. The 0.5 M NaCl eluate contained little protein, but most of the W antigen.

DEAE-cellulose was very effective for the purification of W antigen, as well as the separation of V and W antigen. Before cellulose chromatography the average was 1.0 unit of W/mg of protein and after cellulose chromatography it was 64 units of W/mg of protein. This latter figure was increased to 620 by recycling the W antigen through a small DEAE-cellulose column. Unlike V antigen, W antigen adsorbed strongly to cellulose, and even when a high ratio of protein to cellulose was used, all of the W antigen was adsorbed. Its instability, to be discussed later, probably contributed to the poor recovery in some cases. Only W antigen could be detected when the purified sample was diffused against several complex plague antisera.

Purification of V antigen with DEAE-cellulose was not as satisfactory. The average units of V antigen/mg of protein went from 1.0 before to 3.4 after cellulose chromatography. High percentage recovery was obtained only when the ratio of protein to cellulose was small. For example, with 125 mg of protein/g of cellulose, the recovery in the 0.1 M NaCl eluate was 63%, but with 2800 mg of protein/g of cellulose, the recovery in the 0.1 M NaCl eluate was only 5%. By use of gel diffusion, a minimum of 5 to 7 different antigens were shown to be present in the 0.1 M eluate. Recent experiments, in which the 0.1 M eluate containing V antigen was recycled on DEAE-cellulose and eluted stepwise with 0.06, 0.08 and 0.10 M NaCl, have yielded samples containing 20 units of V antigen/mg of protein with good recovery.

TABLE II

Summary of typical data obtained on the fractionation of V and W antigen on DEAE-cellulose

Experiment No.	Antigen[a]	Units/Mg of Protein		Recovery
		Before	After	
				%
1	V	1.1	3.0	12
	W	2.2	12.3	14
2	V	0.8	5.8	63
	W	3.2	182	13
W recycled	W	182	291	100
3	W	1.3	32	7
W recycled	W	32	620	100
4	V	0.9	2.1	40
	W	0.7	73	60
5	V	1.0	2.7	5
	W	2.1	22	26

[a] In all experiments shown, V antigen was eluted with 0.1 M NaCl and W antigen with 0.5 M NaCl.

Other purification methods. Several different purification possibilities were investigated but discontinued because of their lack of promise. Included among these were calcium phosphate chromatography, continuous flow curtain electrophoresis, precipitation by cold methanol, acid precipitation, Zn^{++} precipitation, fractional solubilization of an ammonium sulfate precipitate by a gradient from 2.5 M ammonium sulfate to distilled water, chromatography on G25 Sephadex, and elution from specific antigen-antibody precipitates. None of these methods improved on the results obtained with ammonium sulfate precipitation and DEAE-cellulose chromatography.

Stability. Both V and W antigen were detected qualitatively after heating purified samples for 30 min at 40°C and 60°C, but not at 80°C or 100°C.

When 25 ml of a culture supernatant fluid was placed in a dialysis bag (45-mm diameter) and dialyzed against 400 ml of distilled water at 5°C with constant agitation by a magnetic stirrer, all of the W antigen, but none of the V antigen, was lost after 2 days. During the 2-day dialysis period, the protein remained at 0.10 mg/ml.

Repetition of this experiment again demonstrated the loss of W antigen. Ten milliliters of a fraction containing 8 units of W antigen/ml were dialyzed against 250 ml of distilled water at 5°C with agitation. After 1 day, only 4 units of W/ml were present, and after 4 days, no W antigen was detected. The 250 ml of dialysate was concentrated to 10 ml and tested in a gel plate, but it contained no detectable W antigen.

The titer of both V and W antigen gradually decreased during prolonged storage at 5°C. For example, one of the best batches produced had a titer of 12 units of V antigen and 24 units of W antigen/ml of supernatant fluid, but dropped to 8 units of V and 16 units of W/ml after storage for 3 days at 5°C. Samples of a greater purity seemed more stable, but it was common to lose 50% of the activity of both V and W antigen during storage for 1 to 2 weeks at 5°C. The effect of storage, dialysis, and lyophilization on a typical preparation of V and W antigen is shown in Table III.

On several occasions both crude and partially purified V and W antigen have been frozen at −20°C without loss. This method is used when a sample is stored more than a few days.

TABLE III

The effect of storage, dialysis and lyophilization on a typical preparation of V and W antigen

Sample	Total Units of Antigen	
	V	W
Culture supernatant fluid	8,300	66,500
Same as (1), incubated at 5°C for 3 days	6,000	32,000
Same as (2), dialyzed overnight	5,800	11,200
Same as (3), lyophilized and resuspended	3,300	7,600

In the methods used to purify V and W antigen, it is essential to concentrate the fractions obtained in order to detect the antigens present. Since 50% of both antigens was lost after lyophilization, other methods of concentrating proteins were tried. Five milliliters of a sample containing 8 units of V and 8 units of W antigen/ml were diluted to 100 ml with distilled water and pervaporated in a cellophane sac in front of a large fan until 5 ml remained. One hundred per cent of the V antigen, but only 25% of the W antigen, was recovered. W antigen could be concentrated 10 to 100 times with no loss by placing the sample in a cellophane sac and surrounding the sac with Carbowax (polyethylene glycol compound 20-M, Union Carbide Chemicals Company, New York 17, New York). This material draws water, but not protein, out of the sac.

Physical and chemical analysis. Three separate molecular weight determinations for each antigen were within the range of 85,000 to 95,000 for V antigen and 140,000 to 150,000 for W antigen. On a dry weight basis, the best V antigen preparation contained less than 1% carbohydrate or nucleic acid and 100% protein. The best W antigen preparation contained 38% lipid and 59% protein. Other purified W antigen preparations contained less than 5% carbohydrate. The lipoprotein nature of W antigen correlates with the recent finding that W antigen (M.W. = 145,000) appeared less dense than V antigen (M.W. = 90,000) when both antigens were sedimented in a sucrose density gradient.

Both antigens were destroyed by trypsin, as judged by loss of ability to react with specific antisera in a gel plate.

Immunization of animals. Injection of 100 units (equivalent to approximately 100 μg) of either antigen intramuscularly in two equal doses spaced 2 weeks apart resulted in no specific antibody formation in mice or guinea pigs when the antigen was mixed 50:50 with incomplete Freund's adjuvant. Mixing was accomplished by syringing the antigen and adjuvant vigorously through a 20-gauge needle until a stable emulsion was formed. Animals were bled approximately 2, 4 and 8 weeks after the last injection. However, when each antigen (same dose and schedule) was mixed 50:50 with Algivant (Colab Laboratories, Inc., Chicago Heights, Illinois), specific antibody formation in guinea pigs to each of the

TABLE IV

Passive protection of mice against plague with rabbit antisera containing V antibody or W antibody

Rabbit Serum[a]	Dead/Total Challenged[b]			
	Expt. 1	Expt. 2		Expt. 3
Normal....	10/10	10/10		10/10
Anti-V[c]....	0/10			0/5
Anti-W[d]....		10/10	5/5	5/5

[a] Five-tenths milliliter of rabbit serum was injected into the peritoneal cavity concomitantly with the challenge dose. Three days later, another 0.5 ml of serum was injected *via* the same route.

[b] Mice were challenged with approximately 1000 *Pasteurella pestis* strain Alexander (100 times the LD_{50} dose) *via* the intraperitoneal route.

[c] Two different antisera were tested.

[d] Three different antisera were tested.

two antigens resulted. Both antigens induced the formation of specific antibody in rabbits when either adjuvant was used. Although the number of animals in the groups was small, the result of challenging the actively immunized guinea pigs indicated that V antigen, but not W antigen, was protective. This result was confirmed by passive immunization studies employing rabbit serum containing either V or W antibody (Table IV). Rabbit anti-V serum protected all the mice when injected into the peritoneal cavity of mice concomitantly with a 100 LD_{50} dose of virulent *P. pestis* strain Alexander. Rabbit anti-W serum or normal rabbit serum showed no protective effect under the same conditions.

DISCUSSION

One of the conclusions to be drawn from this work is the apparent importance of V antibody and the lack of importance of W antibody in protection against plague. The lack of passive protection by W antibody in mice (Table IV) supports the data of Burrows and Bacon (5) that guinea pig antiserum containing measurable amounts of W antibody was nonprotective against *P. pestis* strain M23 challenge in mice.

By use of live purine-dependent strains as vaccines, Burrows and Bacon (5) found V antigen to be poorly antigenic in mice and nonantigenic in guinea pigs. Although our experience in vaccinating animals with purified V antigen supports the impression that V is a poor antigen, results with Algivant indicated that it can induce antibody formation even in guinea pigs. The importance of V antibody would make an investigation into methods of increasing the antigencity of V seem worthwhile.

Unfortunately, V antigen was more difficult than W antigen to purify. One promising possibility of increasing the purity of V antigen is the use of density gradient centrifugation.

Although V and W antigens were always produced together (usually just twice as many units of W as of V), there was no evidence to support the idea expressed by Burrows and Bacon (5) that one antigen may be the precursor of the other. Culture supernatant fluids containing known amounts of each antigen never increased in the concentration of one antigen at the expense of the other.

SUMMARY

The separation and purification of V and W antigens is described. The methods that gave the best results were:

1. The precipitation of both antigens from the supernatant fluid of a 36°C-grown culture of strain M23 by use of ammonium sulfate.
2. Chromatography on DEAE-cellulose.

V antigen was eluted with 0.1 M NaCl and W antigen with 0.5 M NaCl. Recycling on DEAE-cellulose resulted in a sample containing approximately 20 units of V antigen/mg of protein (100-fold purification) and no W antigen, and a sample containing 600 units of W antigen/mg of protein (1000-fold purification) and no V antigen.

V antigen is a protein with a molecular weight of 90,000 and W antigen is a lipoprotein with a molecular weight of 145,000.

Both antigens were stable at 60°C, but not at 80°C, for 30 min. W antigen, but not V, was lost upon extensive dialysis against distilled water, or pervaporation. Both antigens were reduced in titer by prolonged storage at 5°C or by lyophilization, but not by storage at −20°C.

Based on the use of rabbit antisera containing only V antibody or only W antibody, the conclusion was drawn that V antibody, but not W antibody, can protect mice against plague.

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